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Analysis and classification of B-cell infiltrates in lupus and ANCA-associated nephritis

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Intrarenal B cell infiltrates resembling secondary lymphoid tissue have been found in several forms of inflammatory kidney disease. Their role in renal inflammation is not well defined, perhaps because B cell clusters have been regarded as a single entity while being quite heterogeneous. Therefore we characterized intrarenal lymphoid clusters of 32 patients diagnosed with lupus nephritis and 16 with ANCA associated nephritis. We identified four increasingly organized levels of intrarenal aggregates from scattered B cells to highly compartmentalized B cell clusters with central follicular dendritic cell networks. Most B cells displayed a mature non-antibody producing phenotype with antigen presenting ability. In regions of B cell infiltration, expression of the lymphoid chemokine BCA-1 was found in cells of a dendritic-like morphology and most B cells expressed the corresponding receptor CXCR5. Biopsies containing B cells had significantly higher levels of BCA-1 mRNA expression compared to those without, suggesting a role of BCA-1 and CXCR5 for B cell infiltration into the kidney. Our study proposes a new classification of B cell clusters in lupus and ANCA associated nephritis which might help to study the function of intrarenal B cell clusters in a more differentiated manner.

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Recruitment of mononuclear cells into the kidney is a common feature of immunologically mediated renal disease. These infiltrates have long been believed to be composed of mainly monocytes and T cells with little contribution from B lymphocytes. This might be explained by the fact that B cells have classically been considered to exert almost exclusively long-range effects mostly via activation in secondary lymphoid organs with subsequent proliferation and differentiation into antibody-producing plasma cells. However, a number of groups including our own have described the previously unrecognized high prevalence of intrarenal B cells in immunologically mediated diseases, including renal transplant rejection and glomerulonephritis.[1–12] This striking presence in a growing number of renal pathologies has led to many speculations on their functional importance. However, in contrast to the role of T cells[13] and monocytes,[14,15] little attention has been paid so far to renal infiltration of B cells as modulators of inflammatory kidney disease. In many cases, B cells appear in dense cluster-forming units with separate B- and T-cell zones, being reminiscent of secondary lymphoid tissues.[16] It has been speculated that these lymphoid aggregates undergo progressive organization over time[17] and might contribute to the persistence of and therapy resistance in inflammatory processes. In renal transplant rejection, B-cell clusters have been associated with steroid resistance and poorer transplant survival.[2,4–6] Some recent studies reported different results,[18–20] which emphasizes the need for further investigation of the role of intrarenal B-cell clusters.

One reason for these contradictory results could be that the observed clusters, which have so far been regarded as one single entity, are by no means a uniform population. The term lymphoid cluster is rather simplistic, as it has become clear that different grades of microanatomical organization exist in other non-lymphoid tissues.[17,21] Furthermore, some classical features such as follicular dendritic cells (fDCs), high endothelial venules, and germinal centers have not yet been found in the kidney. Patients with highly organized intrarenal clusters might therefore have been grouped with patients with merely nodular B and T cell containing aggregates. No systematic microanatomical analysis of the structure of intrarenal lymphoid tissue has yet been performed. We thus aimed at investigating

the organizational differences between intrarenal inflammatory cell clusters, which could help to clarify the inconclusive results from earlier studies. Furthermore, we investigated the possible contribution of the lymphoid chemokine BCA-1/CXCL13 and its specific receptor CXCR5 to B-cell infiltration and cluster organization in the inflamed kidneys, as this chemokine/chemokine receptor pair has been demonstrated to be an important player in the formation and maintenance of ectopic lymphoid tissue.[22,23] As disease entities, we focused on lupus nephritis (LN) and anti neutrophil cytoplasmic antibodies (ANCA)-associated nephritis (AAN).

RESULTS

Tissue samples

In this study, renal biopsy samples were obtained from 32 patients with LN and from 16 patients with AAN from our center. The diagnosis was based on light microscopy, immunohistochemistry, and electron microscopy. Basic clinical characteristics of the patients are shown in Tables 1a and 1b.

Microanatomical organization of inflammatory infiltrates

For the grading of cellular infiltrates, all biopsy samples from patients with newly diagnosed lupus ($n=32$) and AAN ($n=14$ Wegener's granulomatosis and $n=2$ microscopic polyangiitis) were stained for CD3 as a pan T-cell marker, CD20 as a pan B-cell marker, and CD21 as a marker for fDCs. Thereby, it was possible to identify different organizational levels of intrarenal aggregates that increasingly resembled secondary lymphoid tissue. As B cells have been suggested to be the main cellular players in lymphoid neogenesis,[24] our classification especially highlights their distribution patterns. Scattered B cells and T cells were graded as 1. Nodular aggregates without separate T- and B-cell zones, the second organizational level, were graded as 2. Leukocyte clusters in which distinct T- and B-cell regions had formed were graded as 3. The highest organizational level clearly showed separate B- and T-cell compartments with a central fDC network that is a classical feature of germinal centers of lymph follicles in secondary lymphoid tissue. These aggregates were graded as 4. Many specimens contained lymphocytic aggregates of various grades. Fourteen biopsy samples from patients with LN and four biopsy samples from AAN patients did not contain any B cells and were graded as 0. The remaining 18 lupus and 12 AAN biopsy samples all contained areas of scattered B-cell distribution according to grade 1. Type two clusters without B- and T-cell compartmentalization were found in 10 lupus and 5 AAN specimens. Grade 3 aggregates with distinct B- and T-cell regions but without fDCs were present in three lupus and two AAN biopsy samples. Type

Table 1a | Overview of patients with LN included in the study

Patient no.	Age at biopsy/sex	WHO type	No. of B cells	Grading	Creatinine at biopsy (mg per 100 ml)
1	24/F	V	0	0	0.8
2	26/F	IV	0	0	1.3
3	22/M	IV	0	0	1.0
4	23/F	IV	0	0	1.0
5	31/F	IV	0	0	2.0
6	27/F	II	0	0	3.7
7	23/F	IV	0	0	0.8
8	31/F	IV	0	0	2.0
9	28/F	III	0	0	0.9
10	18/F	IV	0	0	1.3
11	28/F	II	0	0	0.7
12	17/F	III	0	0	0.8
13	47/F	IV	0	0	0.7
14	26/M	IV	0	0	1.6
15	19/F	IV	2	1	2.2
16	53/F	II	5	1	1.3
17	22/F	III	9	1	1.5
18	33/F	II	11	1	1.8
19	65/M	II	25	1	2.2
20	28/F	III	26	1	0.4
21	47/F	IV	36	1	1.0
22	30/M	II	39	1	2.6
23	23/M	III	58	1, 2	3.1
24	24/F	V	85	1, 2	0.7
25	43/M	V	94	1, 2	1.4
26	67/F	III	137	1, 2, 3	2.8
27	35/F	IV	171	1, 2	1.4
28	34/F	IV	194	1, 2, 4	1.8
29	34/F	II	237	1, 2, 3, 4	1.0
30	65/M	IV	259	1, 2, 3	2.5
31	63/M	III	441	1, 2, 4	3.0
32	62/F	V	564	1, 2, 4	0.9

F, female; M, male; WHO, World Health Organization.

Table 1b | Overview of patients with AAN included in the study

Patient no.	Age at biopsy/sex	AAN type	No. of B cells	Grading	Creatinine at biopsy (mg per 100 ml)
33	39/M	WG	0	0	7.6
34	87/F	WG	0	0	4.8
35	24/M	WG	0	0	5.6
36	73/M	MP	0	0	1.4
37	39/M	WG	14	1	1.6
38	59/F	WG	15	1	2.5
39	25/F	WG	18	1	2.6
40	80/M	WG	25	1	4.2
41	59/F	WG	27	1	2.9
42	54/M	WG	29	1	5.8
43	49/M	WG	29	1	2.2
44	63/M	WG	80	1, 2	9.3
45	16/M	WG	97	1, 2	5.6
46	50/M	MP	176	1, 2, 3, 4	2.3
47	41/F	WG	191	1, 2, 3	5.0
48	59/F	WG	209	1, 2, 3	0.8

AAN, ANCA-associated nephritis; F, female; M, male; MP, microscopic polyangiitis; WG, Wegener's granulomatosis.

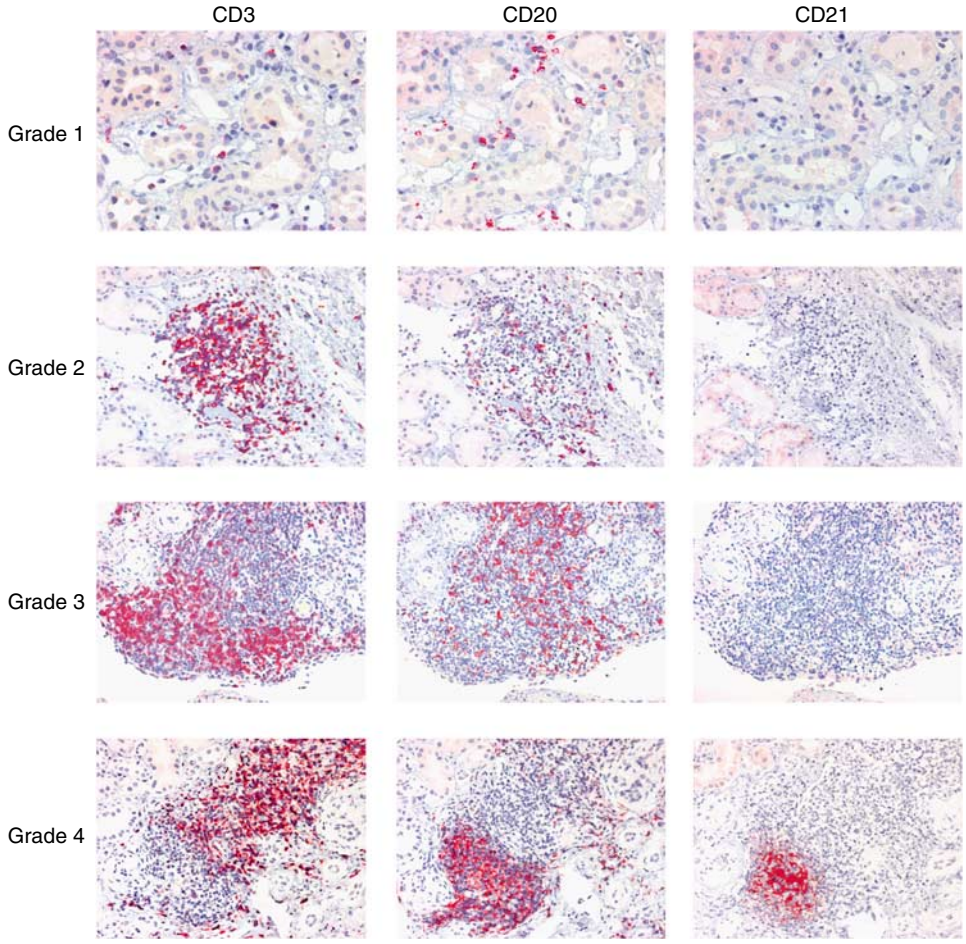

Figure 1 | Microanatomical organization of inflammatory infiltrates. Serial staining for CD3, CD20, and CD21 as a marker for fDCs allowed classification into four different grades. Grade 1 aggregates consist of scattered T- and B-cell infiltrates (all original magnifications $\times$ 400). Grade 2 aggregates show a cluster-like structure but no T- and B-cell zones are evident (original magnification $\times$ 200). In grade 3 aggregates, separate T- and B-cell areas are clearly distinguishable (original magnification $\times$ 100). Grade 4 aggregates show a highly organized structure with a central network of fDCs (original magnification $\times$ 200).

four clusters of the highest organizational level with clearly separate B- and T-cell zones and a central fDC network were found in four patients with LN and in one of the AAN patients. Examples of all four types of organizational levels from patients with LN are given in Figure 1. An overview is given in Tables 1a and 1b.

Characterization of B-cell subpopulations

Little is known about the characteristics of intrarenal B cells. Therefore, we aimed at characterizing different B-cell subsets according to the grade of maturation, antigen-presenting ability, antibody production, and proliferative activity by serial immunohistochemical staining of all biopsy specimens. No CD10-positive pre-B cells were found in infiltrates in LN or AAN (data not shown). CD27, which is presently the best characterized marker for memory B cells[25,26] and also serves as a marker for active T cells, was localized almost exclusively to the T-cell areas in both types of renal diseases (Figure 2a). Staining for IgD, which is regularly present on non-memory B cells (except germinal center B cells) and only rarely found on memory B cells,[27] is shown in Figure 3. B cells outside the fDC containing germinal centers are IgD positive, which identifies them as non-memory B cells. A significant contribution of memory B cells to the infiltrates is therefore unlikely, even though the presence of some cells cannot be ruled out. Staining for the plasma cell marker CD138 showed only positive cells in a scattered distribution in most LN and AAN biopsy samples, whereas almost no positive cells were found within grades 2–4 clusters (data not shown). Proliferative activity was characterized by staining for MIB-1, which showed strong positivity in the central fDC and B cell containing networks in all grade 4 aggregates of LN. All other regions in lupus and AAN, including the single grade 4 cluster found in a specimen obtained from one AAN patient, showed only few MIB-1-positive cells (Figure 2a).

The pan T-cell marker CD5 has been shown to be expressed by B cells of the B1 subset. These specific cells have been reported to be more frequent in the blood of patients with systemic lupus erythematosus[28] and were linked to autoantibody production independent of T-cell activation.[29] However, immunohistochemistry for CD5 showed negative results in B-cell areas and exclusive localization to T-cell regions (Figure 2a).

Staining for CD62L as a marker for high endothelial venules, which are the main routes of entry for lymphocytes into lymph nodes, was negative in all kidney biopsies including grade 4 aggregates (data not shown).

To assess whether B cells could function as intrarenal antigen-presenting cells, we performed serial immunostaining for CD20 and the major histocompatibility complex (MHC) class II complex. MHC class II was abundantly expressed by B cells as clearly seen at higher magnification (Figure 2b) and, in addition, in a scattered distribution in cells with monocyte/macrophage-like morphology. As expected, T-cell regions stained negative for MHC class II (data not shown). Serial immunohistochemical staining of sections from a representative patient with LN is shown in Figure 2a and b. The same results were found in the group of patients with AAN. An example of a grade 4 cluster detected in a sample from an AAN patient is shown in Figure 4. Specificity of all antibodies used was validated in tissues from human tonsils (data not shown).

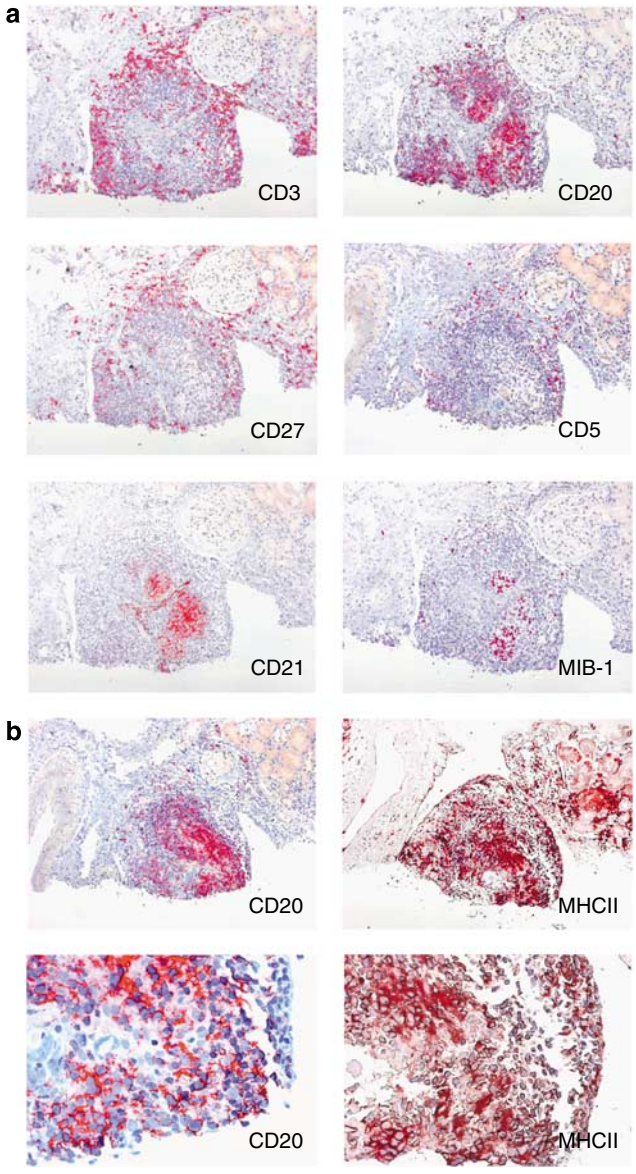

Figure 2 | Immunohistochemical characterization of B cells. (**a**) Serial staining of a grade 4 aggregate shows separate T- (CD3) and B-(CD20) cell zones with a highly proliferating (MIB-1) central fDC (CD21) network. CD27 as a marker for activated T cells, and memory B cells is almost exclusively expressed in the T cell zone. Similarly, CD5 as a marker for T cells and B1 cells is also only found in the T-cell regions (all original magnifications $\times$ 100). (**b**) Serial staining for MHC class II complex shows positivity in B-cell regions (original magnification $\times$ 100). In the higher magnification (original magnification $\times$ 400), B cells can clearly be identified as cellular source for MHC class II expression.

Organization of lymphoid tissue in follow-up biopsies

For three patients with LN (nos. 2, 11, and 23) and one patient with AAN (no. 39), follow-up biopsy specimens were obtained after specific therapy. Interestingly in three of these patients, intrarenal aggregates showed structures of a higher organizational level at the later time points whereas in one patient no change was noted. Infiltrates of the first biopsy specimen from patient no. 2 were graded as 0 whereas grades

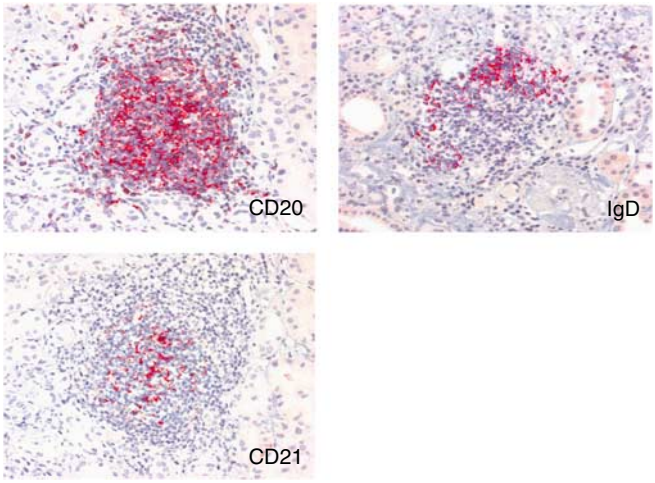

Figure 3 | Germinal centre B cells. Serial staining of a grade 4 aggregate shows that B cells (CD20) inside the CD21-positive fDC containing central network lack IgD expression that is characteristic for germinal center B cells. Furthermore, B cells (CD20) outside the germinal center strongly express IgD, which identifies them as non-memory B cells.

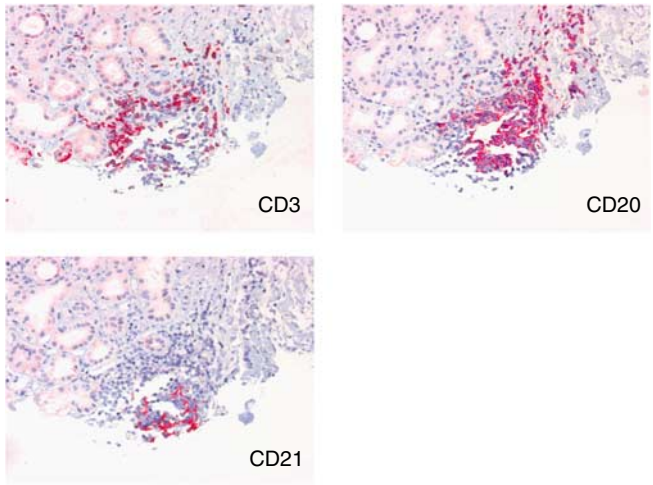

Figure 4 | Grade 4 cluster in AAN. As in lupus nephritis, a grade 4 cluster was also found in AAN that showed the identical microanatomical organization and cellular composition. Clearly, separable T- (CD3) and B-(CD20) cell zones with a central fDC (CD21) network were found (original magnification $\times 200$).

1, 2, 3 and even highly organized grade 4 infiltrates were present in the biopsy specimen obtained 10 years later from the same patient. Similarly, the organization of infiltrates in biopsy specimens from patient no. 11 had progressed from grade 0 to grades 1 and 2 after 2 years. The grading level of intrarenal infiltrates from patient no. 23 remained the same (grade 2 at the first biopsy, grades 1 and 2 at follow-up biopsy 3 years later). In specimens from one patient with AAN (no. 39), the organizational level of infiltrates had progressed from initially grade 1 to grades 2 and 3 aggregates in the follow-up biopsy 6 years later. An overview including treatment modalities is given in Table 2.

Immunohistochemical staining of lymphoid chemokines in renal biopsies

Immunohistochemical staining for the B-cell-specific lymphoid chemokine BCA-1/CXCL13 showed marked colocalization with areas of B-cell infiltration in LN and AAN (Figure 5a and b) specimens. Areas without cellular infiltrates or with substantial infiltration of inflammatory cells negative for CD20 (mainly CD3-positive T cells) did not show any BCA-1/CXCL13-positive staining. As Figure 5c illustrates, the majority of BCA-1/CXCL13-producing cells showed a dendritic cell-like morphology. Staining for CXCR5, the specific receptor for BCA-1/CXCL13, revealed a strong positivity exclusively in regions of CD20-positive B cells in LN and AAN (Figure 6a and b). All other regions were negative for CXCR5. Therefore, a strictly overlapping pattern of CD20-positive infiltrating B cells with BCA-1/CXCL13 and CXCR5 was observed. SLC/CCL21-positive staining was found exclusively in endothelial cells of lymphatic vessels in both types of nephritis. Neither apparent colocalization of SLC/CCL21 with lymphoid aggregates nor cluster forming B cells could be observed (data not shown). To determine specificity of staining, immunohistochemistry in human tonsil tissue was carried out (data not shown).

Intrarenal mRNA expression of BCA-1/CXCL13

To analyze intrarenal expression of the B-cell attracting chemokine BCA-1/CXCL13, RNA was isolated from renal biopsy specimens obtained from the 11 lupus patients with the highest number of B cells (patient nos. 22–32) and 11 lupus patients devoid of intrarenal B cells (patient nos. 3, 4, 6, 8–15). mRNA analysis revealed a 107.7 ± 34.1-fold higher

Table 2 | Characterization of follow up biopsies

Patient no.	Nephritis type first/ follow-up biopsy	Grading at first biopsy	Grading at follow-up biopsy	Time to follow-up biopsy (months)	Treatment between biopsies	Creatinine at first biopsy (mg per 100 ml)	Creatinin at follow-up biopsy (mg per 100 ml)
2	IV/IV	0	1, 2, 3, 4	116	CYC, CSA, MMF, Pred	1.3	0.8
11	II/IV	0	1, 2	23	No treatment	0.7	7.4
23	III/IV	1, 2	1, 2	36	CYC, CSA, AZA, MMF, PP, Pred	3.1	9.8
39	WG/WG	1	2, 3	73	CYC, Pred	2.6	5.0

AZA, azathioprine; CSA, cyclosporine A; CYC, cyclophosphamide; MMF, mycophenolate mofetil; PP, plasma separation; Pred, prednisone; WG, Wegener's granulomatosis.

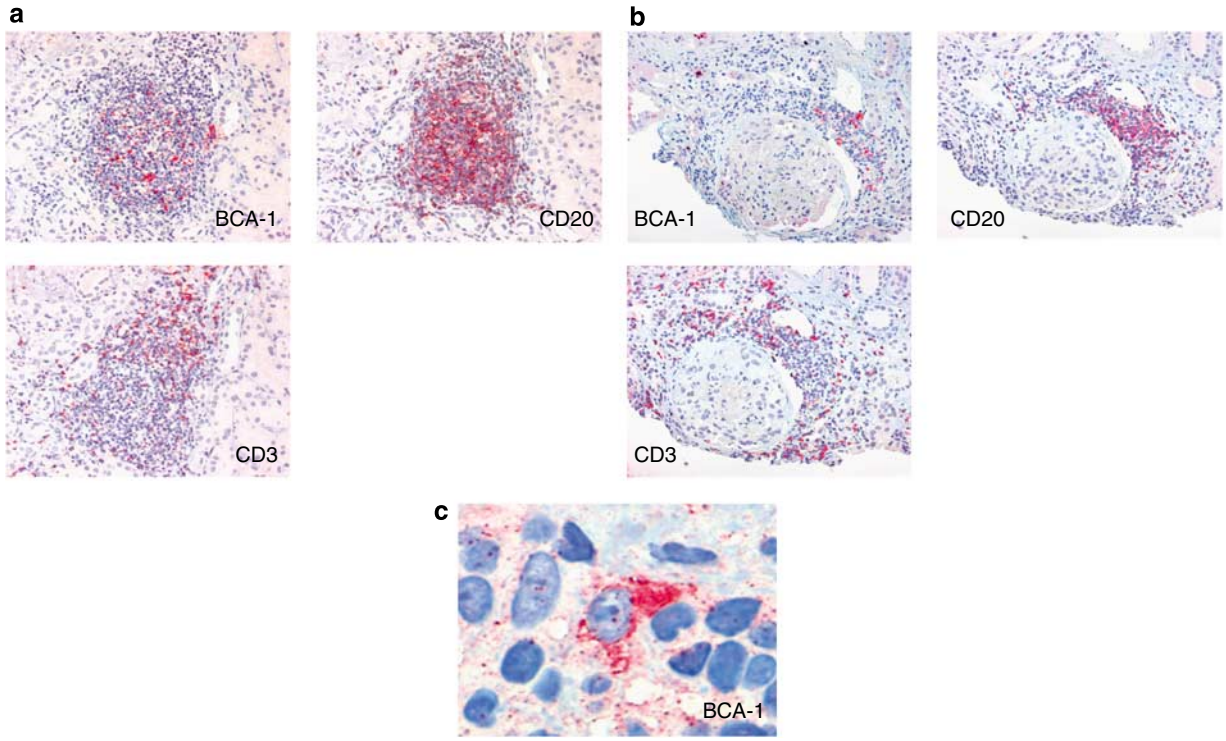

Figure 5 | Intrarenal expression of BCA-1/CXCL13. Serial staining for B cells (CD20), T cells (CD3), and the lymphoid chemokine BCA-1/CXCL13 showed exclusive BCA-1 expression in B-cell areas for both lupus nephritis (**a**) and AAN (**b**) (all original magnifications $\times$ 200). (**c**) A BCA-1-producing cell with typical dendritic morphology is shown (original magnification $\times$ 1000).

renal BCA-1/CXCL13 expression in the group of lupus patients with abundant intrarenal B cells compared with the remaining 11 patients without intrarenal B cells ($P<0.01$) (Figure 7a). Similarly, mRNA was isolated from the six AAN patients with the highest B-cell count (patient nos. 43–48) as well as from the six patients with the lowest number of intrarenal B cells (patient nos. 33–38). In line with the observation in LN, mRNA analysis showed a significantly higher BCA-1/CXCL13 expression (83.5 ± 25.7-fold, $P<0.01$) in B-cell-rich biopsy specimens compared with those that contained no or only few B cells (Figure 7b).

DISCUSSION

In the last couple of years, numerous authors, including our own group, have described the previously unrecognized presence of intrarenal B cells in immunologically mediated renal diseases, such as transplant rejection and glomerulonephritis.[1–12] These B cells are exclusively detectable in the tubulointerstitial compartment, but not in the glomerular tuft.[30] Many questions have arisen from these observations. It would clearly be interesting to clarify the functional importance of intrarenal B cells and the mechanisms leading to their tissue infiltration. Because no functional data from animal studies are available, several groups have correlated the presence of intrarenal B cells with clinical outcome measures such as serum creatinine or steroid resistance in renal transplant rejection. The results of these retrospective studies were inconclusive. Some investigators found deleterious effects associated with intrarenal B cells in renal transplant rejection,[2,4–6] whereas others regarded the presence or absence of B cells as irrelevant.[18–20] These conflicting results might on the one hand be explained by the small numbers of patients included in the studies and their retrospective character. However, one important aspect of intrarenal B cells has not yet been investigated at all, namely the grade of microanatomical organization. In previous studies, inflamed kidneys were classified as containing no B cells, B cells in a scattered pattern or B cells in the form of clusters. This form of classification may not be accurate enough because different organizational levels might have different clinical implications. Therefore, our present morphological study was aimed to characterize B-cell infiltrates in lupus and AAN and to classify the infiltrates according to their microanatomical structures. Characteristic and functionally important hallmarks of lymph follicles in secondary lymphatic tissues are distinct and separate T- and B-cell zones and a central network of fDCs. To assess potential similarities of intrarenal aggregates that contain these follicles, we performed immunostaining using the pan B-cell marker CD20, the pan T-cell marker CD3, and CD21 as a marker for fDCs. Analysis of 32 biopsy specimens from patients with LN and 16 patients with AAN revealed four different subgroups of infiltrates with increasingly organized structures. Whereas scattered B-cell infiltrates (grade 1) were mainly found in the renal cortex but occasionally also seen in the medulla, grades 2–4 aggregates were exclusively found in the cortex. The

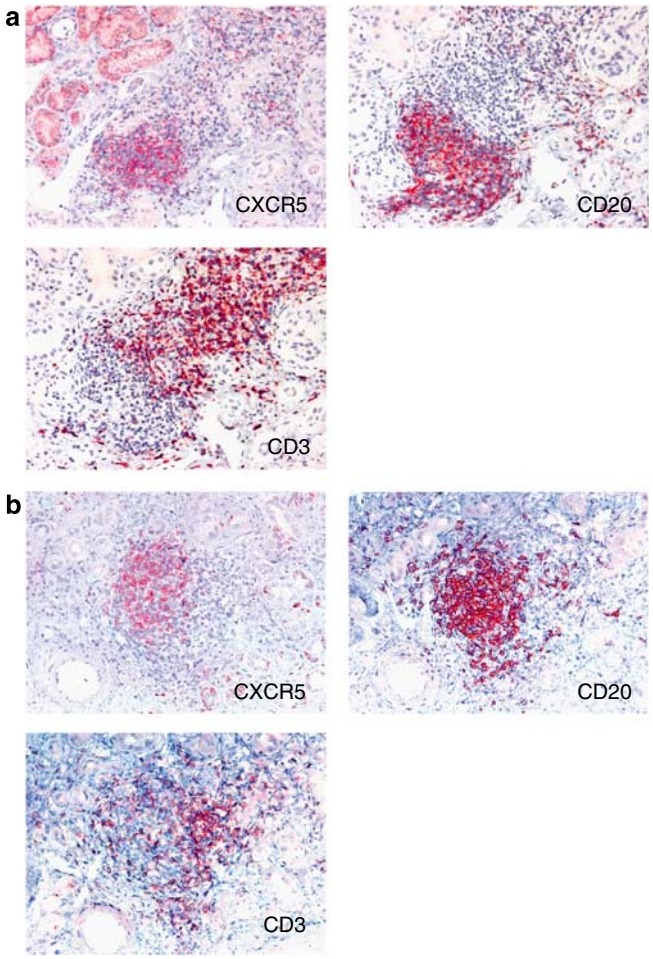

Figure 6 | Expression of chemokine receptor CXCR5. Serial staining for B cells (CD20), T cells (CD3), and the chemokine receptor CXCR5 showed exclusive CXCR5 expression in B-cell areas for both lupus nephritis (**a**) and AAN (**b**) (all original magnifications $\times$ 200).

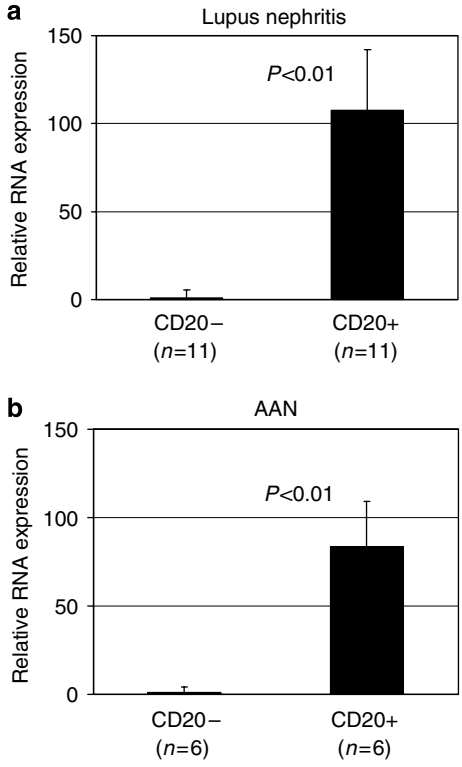

Figure 7 | Quantification of intrarenal BCA-1/CXCL13 mRNA expression. Analysis of intrarenal BCA-1/CXCL13 mRNA expression by real-time PCR from formalin-fixed biopsies revealed a massive upregulation in B-cell containing biopsies as compared with those without (**a**: 107.7 ± 34.1-fold for lupus nephritis and **b**: 83.5 ± 25.7-fold for AAN, both $P < 0.01$).

highest organizational grade seen in four lupus biopsy specimens and in one AAN kidney contained clearly separate T- and B-cell zones with a central fDC network. In less-organized aggregates (grades 1–3), only little proliferation was detected by staining for MIB-1. This argues for infiltration of B cells from the circulation into the kidney. However, a proliferating center around a network of fDCs was found in clusters of the highest organizational level (grade 4). Unlike B cells surrounding the proliferating center, the central B cells did not express IgD. This exactly mirrors the situation in germinal centers of lymphatic organs in which B cells lose IgD expression upon entry into the germinal center. Here B cells undergo affinity maturation, differentiation, and proliferation. Our data therefore provide evidence for a local, intrarenal outpost of the immune system that makes it unnecessary for leukocytes to leave the kidney and migrate toward the lymph nodes and spleen. Local proliferation in these germinal centers might lead to the expansion and perpetuation of inflammatory cell aggregates that could result in persistence and chronicity of the renal inflammation.

As it is completely unclear via which routes lymphocytes enter the observed intrarenal lymphatic aggregates, we performed immunostaining for CD62L, which is a marker for high endothelial venules. These highly specialized vessels are specific routes of entry for lymphocytes into lymph nodes and could therefore also play a role in the entry of lymphocytes into the kidney. However, no CD62L-expressing venules were found, arguing for different, still unknown mechanisms.

For further examination of the possible mechanisms that might lead to B-cell cluster formation, we performed immunohistochemical staining of biopsy samples for BCA-1/CXCL13. The chemokine BCA-1/CXCL13, which acts via the chemokine receptor CXCR5 on B cells, is one of the most potent B-cell chemoattractants[31] and is one of the key players in lymphoid organogenesis.[32–34] We previously showed that BCA-1/CXCL13 was associated with the formation of B-cell clusters in acute interstitial and vascular transplant rejection.[4,12] Here, we could demonstrate a strong BCA-1/CXCL13 expression that is exclusively localized to regions of B-cell infiltration irrespective of the organizational level. Areas without CD20-positive B cells stained completely negative for BCA-1/CXCL13. Similarly, positivity for the corresponding receptor CXCR5 was specifically found in B-cell regions, whereas all other resident and infiltrating cells

stained negative. Analysis of RNA from sections of formalin-fixed biopsy specimens underlined the association of BCA-1/CXCL13 with B cells. Sections with high B-cell numbers from patients with LN contained 107.7-fold more BCA-1/CXCL13 RNA in comparison with biopsies from LN patients without B-cell infiltration and B-cell-positive sections from AAN patients contained 83.5-fold more BCA-1/CXCL13 as compared with the B-cell-negative group. It is therefore likely that locally produced BCA-1/CXCL13 plays a functional role in the infiltration of CXCR5-bearing B cells in LN and AAN.

The second chemokine, which is known to be crucially implicated in lymphocyte homing to lymphoid tissues, is SLC/CCL21 acting via its receptor CCR7.[33,34] SLC/CCL21-positive lymphatic vessels were present in high numbers in biopsies of all grades. They were not only found in the proximity of B-cell clusters but also in regions without B-cell infiltration. Therefore, as Kerjaschki *et al.* and our own group[3,4,12] have previously shown in acute transplant rejection, newly formed SLC/CCL21-positive lymphatic vessels are widely distributed in the inflamed kidney but are not specifically associated with B cells. The role for SLC/CCL21 is still unclear and rewards further investigation.

Another aspect that has not yet been examined in detail is the characterization of intrarenal B-cell subgroups with regard to their grade of maturation, their ability for antibody production, and antigen presentation. Our studies revealed the absence of CD10-positive pre-B cells and only sparse presence of CD27-positive and IgD-negative memory B cells in LN and AAN specimens of all microanatomical levels of organization. Plasma cells were also almost totally absent from clusters of grades 2–4 and were usually found in a scattered distribution in both, LN and AAN. Another possible function of intrarenal B cells, especially those in cluster-forming units that are in close proximity to numerous other leukocyte subsets, is antigen presentation and activation of surrounding immune cells such as T cells. The MHC class II receptor is characteristic of cells that are professional antigen presenters. These professional antigen-presenting cells take up antigens very efficiently and use their MHC class II receptors to present processed antigen fragments to CD4-positive helper T cells, which are thereby activated. All other cells, including T cells, do not express MHC class II receptors but use MHC class I instead to present antigen that cannot activate T-helper cells. Staining for the MHC class II complex revealed strong positivity in intrarenal B-cell regions. This finding has not been described before and may be seen as an indicator of the function of intrarenal B cells as antigen presenters.

In several previous studies, a distinct subset of B cells, termed B-1 cells, which are positive for the pan T-cell marker CD5, has been identified. This specific subgroup was shown to be more frequent in the blood of patients with systemic lupus erythematosus[28] and was linked to autoantibody production independent of T-cell activation.[29] Furthermore, aberrant homing of B1 cells to inflamed organs, including the kidneys, has been shown in a mouse model of systemic lupus erythematosus.[35] To test whether these cells are present in the kidneys, we performed CD5 staining. Our analysis, however, showed positive staining only in T-cell areas, whereas no signal was seen in B cell areas. These results show that the majority of intrarenal B cells, regardless of the organizational grade, are of the mature B2, non-antibody-secreting phenotype with antigen-presenting ability.

To test whether the observed lymphoid aggregates undergo progressive organization over time by which, for example, a grade 2 aggregate could progress to a grade 4 aggregate, we analyzed follow-up biopsies from three patients with LN and one patient with AAN. Interestingly, in spite of immunosuppressive therapy in three cases, the aggregates had not resolved in any of the four patients. In three of these four patients, aggregates of higher organizational levels were present at the later time points, whereas the aggregates found in the fourth patient after therapy were still of the same grades as before. These findings suggest a progressive organizational process of intrarenal lymphatic structures over time. The currently available immunosuppressants do not seem to have a sufficient effect on formation of intrarenal ectopic lymphoid tissue. This might be one of the reasons for chronic manifestation and recurrent flares of LN and AAN.

It would be very interesting to study correlations between clinical parameters and the presence of the four different intrarenal lymphoid aggregates discussed here. However, to do this, a larger number of patients than included in this study would be needed to ensure sufficient statistical power.

In summary, our study revealed the presence of intrarenal lymphoid structures of four different organizational stages. In spite of immunosuppressive therapy, these aggregates did not resolve but instead showed higher organizational levels. The most organized aggregates closely resemble secondary lymphatic tissue with fDC networks and proliferative centers. Characterization of contributing B-cell populations revealed that most of the B cells in all cluster types were of a mature, non-antibody-secreting phenotype with the ability of antigen presentation and helper T-cell activation via MHC class II receptors. Their distribution was strongly correlated with intrarenal expression of the lymphoid chemokine BCA-1/CXCL13 by cells of a dendritic cell-like morphology and most B cells were positive for the corresponding receptor CXCR5. A role for this chemokine/chemokine receptor pair in B-cell infiltration is therefore likely. The clinical impact of this intrarenal lymphoid tissue is still unclear. However, our proposal for a new and more detailed classification of intrarenal lymphoid clusters might help to explain some of the contradictory results from previous reports and could help to shed some more light on the function of intrarenal B cells in future studies.

MATERIAL AND METHODS

Patients and tissue samples

The study was conducted in accordance with the Declaration of Helsinki Principles and with approval from the local ethics committee. Renal biopsy samples were obtained from the archive of the Department of Pathology of the University

Hospital of Hamburg Eppendorf. Only patients in whom systemic lupus erythematosus or AAN (Wegener's disease or microscopic polyangiitis) had been diagnosed in our center between 1992 and 2003 were included in the study. The same biopsy specimens were used for immunohistochemistry and preparation of mRNA. Clinical classification of renal pathologies was carried out by one single nephropathologist. Follow-up biopsies after specific treatment were available in three cases with LN and in one case with AAN. Clinical data of the patients were obtained retrospectively from the records of our center.

Immunohistochemistry

The following antibodies were used for immunohistochemical staining: CD20 (L26, Dako; Glostrup, Denmark), CD3 (N1580; Dako), MIB-1 (dia 505; Dianova, Hamburg, Germany), CD138 (VS38c; Dako), IgD (A0093; Dako), BCA-1/CXCL13 (AF801; R&D System, Wiesbaden, Germany), SLC (AF 366; R&D System), CXCR5 (51505; R&D System), CD10 (56C6; Dianova), CD62L (MEKA-79, Becton Dickinson, Heidelberg, Germany), CD5 (4C7; Novocastra, Newcastle, UK), MHC class II (CR3/43; Dako), CD21 (1F8; Dako), and CD27 (137B4; Dianova).

Formalin-fixed, paraffin-embedded tissue sections (2 μm) were deparaffinized and rehydrated through graded ethanol solutions. Antigen retrieval for staining with CD3, CD5, CD10, CD20, CD62L, BCA-1, SLC, and CD138 was achieved by placing sections in citrate buffer (pH 6.1) and heating in the microwave for 25 min. In the case of IgD, sections were placed in citrate buffer at pH 9.0 and heated in the microwave for 10 min. For staining with CD21, CD27, and CXCR5, samples were pre-treated in an autoclave for 15 min. For staining with the MHC class II antibody, sections were pre-treated with protease for 30 min. Sections were incubated with most primary antibodies for 30 min with the exception of CD10, CXCR5, and BCA-1 with which biopsies were incubated overnight. Tissue sections were developed with the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA, USA) as described.[36]

Grading of biopsies

Renal biopsies were classified according to the organizational stage of inflammatory cell infiltrates. Four distinct categories could be distinguished. A scattered pattern of intrarenal CD20+ B cells was graded as 1. Nodular aggregates consisting of CD3-positive T cells and B cells without microanatomical compartmentalization were graded as 2. In the case of clearly separated T-cell and B-cell zones without a central dendritic cell network, aggregates were classified as 3. Aggregates with the highest level of microanatomical organization, consisting of separated T- and B-cell compartments with a central network of CD21+ fDCs were classified as 4. Biopsies with no intrarenal CD20+ B cells were graded as 0.

Preparation of total RNA from formalin-fixed tissue

For preparation of total RNA, the High Pure FFPE RNA kit (Roche, Mannheim, Germany) was used. Two 10-μm-thick sections from archived formalin-fixed and paraffin-embedded tissue samples were placed in a 1.5-ml reaction tube. Deparaffination was carried out by incubation in Xylol with subsequent washing steps in 100% and 70% ethanol. Lysis of the tissue pellet was then carried out by adding 60 μl of the specific lysis buffer with 10 μl of 10% sodium dodecyl sulfate. Afterward, 30 μl of Proteinase K were added and the probes were incubated for 3 h at 55 °C. After centrifugation, 200 μl of binding buffer and 200 μl of 100% ethanol were added and the lysate was allowed to bind to the specific High Pure Micro Filter Tube assembly. Hereafter, the columns were incubated for 15 min at room temperature in DNase solution followed by three washing steps. The final elution of total RNA was carried out in a volume of 30 μl elution buffer.

Real-time PCR analysis

RNA of the formalin-fixed biopsy tissue was isolated as described above. Total RNA was reverse transcribed using the Taqman Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis was performed in the ABI-Prism 7000 Sequence Detection System (Applied Biosystems) as described before.[36] The following human PCR primers were used:

hBCA-1.3: GCAGCCTCTCTCCAGTCCAA
hBCA-1.4: TGGACACATCTACACCTCAAGCTT

All specific amplicons were normalized against ribosomal RNA (18 s).

Statistical analysis

Data are presented as mean ± s.d. Relative expression of BCA-1/CXCL13 in the two groups was compared by the Mann–Whitney *U*-test. All statistical analyses were carried out using the SPSS 10.1 software.

DISCLOSURE

None of the authors has relationships with any company that has a financial interest in the information contained in the manuscript.

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